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Direct Interspecies Electron Transfer between *Geobacter metallireducens* and *Methanosarcina barkeri*

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Direct interspecies electron transfer (DIET) is potentially an effective form of syntrophy in methanogenic communities, but little is known about the diversity of methanogens capable of DIET. The ability of *Methanosarcina barkeri* to participate in DIET was evaluated in coculture with *Geobacter metallireducens*. Cocultures formed aggregates that shared electrons via DIET during the stoichiometric conversion of ethanol to methane. Cocultures could not be initiated with a pilin-deficient *G. metallireducens* strain, suggesting that long-range electron transfer along pili was important for DIET. Amendments of granular activated carbon permitted the pilin-deficient *G. metallireducens* isolates to share electrons with *M. barkeri*, demonstrating that this conductive material could substitute for pili in promoting DIET. When *M. barkeri* was grown in coculture with the H_2-producing *Pelobacter carbinolicus*, incapable of DIET, *M. barkeri* utilized H_2 as an electron donor but metabolized little of the acetate that *P. carbinolicus* produced. This suggested that H_2, but not electrons derived from DIET, inhibited acetate metabolism. *P. carbinolicus-M. barkeri* cocultures did not aggregate, demonstrating that, unlike DIET, close physical contact was not necessary for interspecies H_2 transfer. *M. barkeri* is the second methanogen found to accept electrons via DIET and the first methanogen known to be capable of using either H_2 or electrons derived from DIET for CO_2 reduction. Furthermore, *M. barkeri* is genetically tractable, making it a model organism for elucidating mechanisms by which methanogens make biological electrical connections with other cells.

Effective interspecies electron transfer is key to the efficient functioning of methanogenic communities (1–4). Promoting interspecies electron transfer to methanogens enhances the anaerobic digestion of wastes, and appropriate models of the pathways for interspecies electron transfer are necessary in order to predictively model the response of methanogenic communities to environmental change.

The best-known strategy for interspecies electron transfer is H_2 interspecies transfer (HIT), in which electron-donating microorganisms reduce protons to H_2 and methanogens oxidize the H_2 with the reduction of carbon dioxide to methane (1). In some instances, formate substitutes for H_2 as the electron carrier (2–6). The abundance of H_2/formate-consuming methanogens in anaerobic soils (7, 8) and sediments (9, 10) as well as some anaerobic digesters (11) suggests that HIT plays an important role in methane production in those environments. HIT has been documented in studies with defined cocultures of H_2-donating microorganisms and H_2-consuming methanogens, and the physiology and biochemistry of both H_2 production and H_2 consumption are relatively well understood (1, 12, 13).

Direct interspecies electron transfer (DIET) is an alternative to HIT. *Methanosaeta (Methanotrix) harundinacea*, which is representative of the *Methanosaeta* species, which are responsible for a substantial portion of the global methane production (14), directly accepted electrons from *Geobacter metallireducens* for the reduction of carbon dioxide to methane in defined cocultures (15). DIET in these cocultures required that *G. metallireducens* produce pili (15), that have a metal-like conductivity (16, 17), but further details of the interspecies electrical connections are as yet unknown. Multiple lines of evidence suggested that *Methanosaeta* species were also participating in DIET in anaerobic digesters treating brewery wastes (15, 18). *Geobacter* species were abundant in the digester granules, which possessed a metal-like conductivity (18) similar to the metal-like conductivity of *Geobacter* pili (16–18). The potential importance of conductive pili in electron transfer to *Methanosaeta* species is consistent with the clear importance of conductive pili in DIET in cocultures of *G. metallireducens* and *G. sulfurreducens*, cooperating to oxidize ethanol with the reduction of fumarate (19–22). The electrical connections that *M. harundinacea* employs for DIET are unknown, and elucidating them may be technically difficult because a system for genetic manipulation of this organism has not yet been identified.

Another genus of acetoclastic methanogens, *Methanosarcina*, is often abundant in methanogenic soils and sediments (23–25), coal mines (26, 27), landfills (28), and anaerobic digesters (29, 30). Several studies suggested that *Methanosarcina* species could accept electrons from nonbiological extracellular surfaces (31, 32). For example, *M. barkeri* attached onto electrically conductive granular activated carbon (GAC), which served as a mediator for electron transfer between *M. barkeri* and *G. metallireducens* (32). Conductive iron minerals were proposed to function as mediators between *Geobacter* and *Methanosarcina* species in a similar manner, based on the abundance of these genera in mineral-amended enrichment cultures (31). Here we provide evidence that *M.*

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barkeri does not require a conductive mediator for DIET because it is capable of forging direct biological electrical connections to receive electrons from *G. metallireducens*.

MATERIALS AND METHODS

Strains and conditions for cultivation. *Methanosarcina barkeri* (DSM 800) and *Pelobacter carbinolicus* (DSM 2380) were purchased from the DSMZ culture collection. *Geobacter metallireducens* strain ATCC 53774 as well as gene deletion strains in which either the gene for PilA, the structural pilin protein (Gmet 1399) gene (33), or the Gmet 1868 gene, encoding a putative outer surface *c*-type cytochrome (34), was deleted were obtained from our laboratory culture collection. Strict anaerobic culturing procedures with either pressure tubes or serum bottles sealed with thick butyl rubber stoppers were used throughout.

M. barkeri was grown on 30 mM acetate in a modification of DSM 120 medium, which was determined to also support good growth of *G. metallireducens*. The DSM 120 modifications were as follows: 0.5 mM sulfide, 1 mM cysteine, 0.002 g/liter $CaCl_2 \cdot 2H_2O$, 1 g/liter NaCl, no yeast extract, no Casitone, no resazurin, and 2 g/liter $NaHCO_3$.

G. metallireducens wild-type and mutant strains were routinely cultured on a ferric citrate medium amended with 10 mM acetate (33). However, prior to initiating cocultures with *M. barkeri*, the *G. metallireducens* strains were adapted for at least three transfers (5 to 10% inoculum) in ferric citrate medium in which the acetate was replaced with 20 mM ethanol.

P. carbinolicus was routinely grown on 10 mM acetoin, as previously described (20).

The coculture inocula were obtained from cultures that were in late exponential or early stationary phase, as determined by monitoring methane production (*M. barkeri*), Fe(II) production (*G. metallireducens*), or the optical density at 600 nm (OD_{600}) (*P. carbinolicus*).

The *G. metallireducens-M. barkeri* cocultures were initiated with a 0.5-ml inoculum of the ethanol-adapted *G. metallireducens* and a 0.5-ml inoculum of an acetate-grown *M. barkeri* culture added to 9 ml of the modified DSM 120 medium described above, but with the acetate replaced with 20 mM ethanol as the electron donor. CO_2 or HCO_3^- was the only potential electron acceptor. After the coculture was established, it was routinely transferred into 45 ml of medium with a 10% inoculum. Coculture growth was also tested in the absence of sulfide and cysteine, but for long-term incubations, both sulfide and cysteine are required as sulfur sources for *M. barkeri* (35–37) and were therefore added consistently to the coculture medium. When noted, GAC was added as previously described (32). Similar to GAC-free cocultures, 9 ml medium with GAC was inoculated with 0.5 ml of a *G. metallireducens* strain culture and 0.5 ml of *M. barkeri* culture.

M. barkeri grew in the same medium as *P. carbinolicus*, but *P. carbinolicus* could not grow in DSM 120 medium or the modified version of it. Therefore, the *P. carbinolicus-M. barkeri* cocultures were initiated with a 5% inoculum of acetoin-grown *P. carbinolicus* and a 5% inoculum of acetate-grown *M. barkeri* in the previously described *P. carbinolicus* medium (20). After establishing the coculture, *P. carbinolicus* and *M. barkeri* were routinely transferred together using 10% inocula.

Analytics. Headspace methane (0.5 ml) was sampled at regular intervals using strict anaerobic sampling techniques (38), injected on a Shimadzu 8A gas chromatograph (GC) equipped with an 80/100 Hayasep Q column heated at 110°C. The injector port and flame ionization detector (FID) were both set at 200°C (15). To determine the concentration of organic acids and ethanol, 200 μl of culture medium was sampled, diluted, and placed in tightly sealed vials specially designed for high-performance liquid chromatography (HPLC) analysis (for fatty acids) and GC analysis (for ethanol). Organic acid samples were analyzed immediately or stored at 4°C for a maximum of 1 week. HPLC analyses were performed as previously described (39) by separating the organic acids on an Aminex NPX-87H column using 8 mM H_2SO_4 as the eluent. Organic acids were detected at 210 nm by a UV detector. Under these conditions, the following organic acids could be monitored: acetate, formate, succinate, fumarate, lactate, propionate, and butyrate. The detection limit for all was ca. 5 μM. In all our samples, only acetate was detected. Gas chromatography analyses of ethanol were performed on a PerkinElmer Clarus600 GC equipped with a headspace automatic sampler and an FID. Separation of ethanol was obtained on an Elite 5 (PerkinElmer) column (30-m length, 0.25-mm inner diameter) using He as the carrier gas and the following separation parameters: 50°C for 1 min, a ramp of 12°C per minute to reach 200°C, and 1.5 min at 200°C. The injector and detector temperatures were set at 200°C and 300°C, respectively. The chromatography data from GC and HPLC analyses were analyzed with an integrated TotalChrom data analysis system.

The increase in aggregate biomass over time was determined as previously described (40) by harvesting (15 min, 3,600 × *g*) 50 ml of three *M. barkeri-G. metallireducens* cocultures at different growth stages: during the initial growth phase (day 5) and in stationary phase (day 28). Aggregates were washed in isotonic buffer and freeze-dried for 48 h on a Labconco lyophilizer at −50°C. The weighted dry biomass was solubilized in 0.5% SDS by steaming. The total protein in the steamed biomass was determined using the Pierce bicinchoninic acid (BCA) protein assay kit according to the manufacturer's instructions. Bovine serum albumin was used as a protein standard.

Microscopy. To evaluate cell-to-cell contact, we checked unfixed cells immediately after removing them from a culture tube and visualized the cultures with phase-contrast microscopy on a Leica Axioplann microscope. The presence of the methanogens was verified by fluorescence at 420 nm and identification of their specific coccus shape. To better visualize how cells were distributed within the aggregates, we performed fluorescence *in situ* hybridization. Cells were sampled in mid-log phase, fixed in premixed glutaraldehyde (0.5%) and paraformaldehyde (1%), rinsed in 50 mM PIPES [piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)] buffer, dehydrated (30 min at 4°C in an ethanol series at 30%, 50%, 70%, 80%, and 100%), and preembedded in a 50:50 mix of ethanol (100%) and butyl-methyl-methacrylate (BMM). Ultimately, aggregates were embedded in BMM resin and polymerized on a Spectorlinker cross-link XL-1000 instrument at room temperature overnight. Sections of the aggregates were obtained by microtome and stained with Cy3 probes (red) targeting *Methanosarcina* species (MS1414, 5′-CTCACCCATACCTCACTCGGG-3′) (41) and Cy5 probes (green) specific for the *Geobacter* cluster (Geo3ABC, 5′-CCGCAA CACCTRGTWCTCATC-3′) (42) or Cy5 (green)-labeled probes specific for *P. carbinolicus* (PCARB1, 5′-GCCTATTCGACCACGATA-3′) (42) depending on the coculture. The labeled cocultures were visualized using a confocal laser microscope as previously described (20).

RESULTS AND DISCUSSION

Growth of *M. barkeri* via DIET with *G. metallireducens*. *Methanosarcina barkeri* was cocultured with *Geobacter metallireducens* in a medium with ethanol as the sole electron donor in order to determine whether it was capable of DIET. Multiple lines of evidence have indicated that *G. metallireducens* is unable to release the electrons derived from the oxidation of ethanol to acetate as either H_2 or formate but can directly transfer these electrons to microorganisms such as *G. sulfurreducens* or *Methanosaeta harundinacea*, which are capable of DIET (15, 19–21). After a lag of ca. 39 days, cocultures of *G. metallireducens* and *M. barkeri* started metabolizing ethanol to methane (Fig. 1a). Neither *G. metallireducens* nor *M. barkeri* metabolized ethanol in pure culture (Fig. 1b). With continued transfer of the coculture, the initial long lag in ethanol metabolism was eliminated, and ethanol began to be metabolized to methane in less than 7 days (Fig. 1c).

Acetate only transiently accumulated in the cocultures (Fig. 1a and c). The total ethanol consumed (mean ± standard deviation, 0.95 ± 0.01 mmol; $n = 4$) resulted in 1.59 ± 0.04 mmol methane, consistent with the expected production of 1.5 mol of methane for

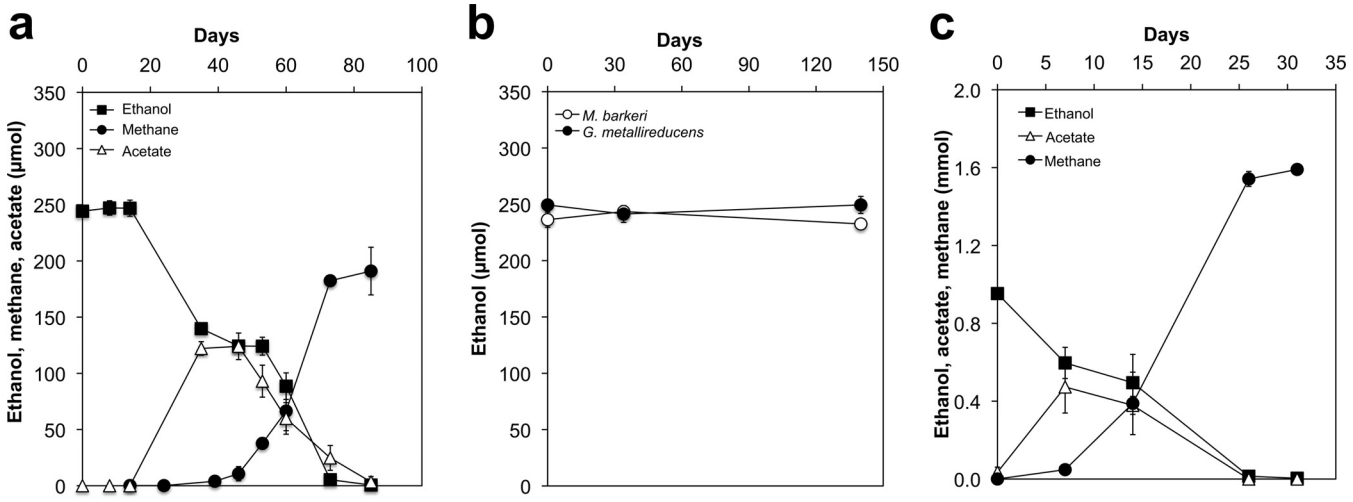

FIG 1 Syntrophic growth of *M. barkeri* with *G. metallireducens*. (a) Metabolism of ethanol when the cocultures were initiated with a 5% (vol/vol) inoculum of each species. (b) Lack of ethanol metabolism with single species. (c) Decreased lag after 25 transfers (10% inoculum) of the cocultures. All cultures were grown in triplicate ($n = 3$). A difference in the *y* axis scale can be noted between panels a and b and panel c. This is because of differences in the total incubation volumes: 10 ml to initiate the cocultures (a, b) and 50 ml for later incubations (c).

each mole of ethanol consumed according to the following equation: $2\ C_2H_6O + H_2O \rightarrow 3\ CH_4 + CO_2$.

This stoichiometric conversion of ethanol to methane requires that *M. barkeri* not only metabolize the acetate that *G. metallireducens* produces from ethanol ($2\ C_2H_6O + 2\ H_2O \rightarrow 2\ C_2H_4O_2 + 8\ H^+ + 8\ e^-$ via the reaction $2\ C_2H_4O_2 \rightarrow 2\ CH_4 + 2\ CO_2$) but also use the electrons released from ethanol metabolism to reduce carbon dioxide to methane: $8\ H^+ + 8\ e^- + CO_2 \rightarrow CH_4 + 2\ H_2O$. Thus, the high electron recovery of electrons available from ethanol in methane and the fact that *G. metallireducens* is incapable of producing H_2 during ethanol metabolism (22) strongly suggested that *M. barkeri* was accepting electrons for carbon dioxide reduction via DIET.

The fact that the coculture could be continually transferred (10% inoculum) indicated that a small fraction of the carbon and electrons derived from ethanol must also be consumed for biomass production. Biomass was examined by sacrificing triplicate cocultures for protein analysis at day 5, when methane started increasing, and at day 28, when methane production was complete. Protein increased from 233 ± 62 μg ml^{-1} (mean ± standard deviation) to 861 ± 115 μg ml^{-1}, verifying coculture growth.

Cells involved in DIET need physical connections. *G. metallireducens* and *M. barkeri* cocultures formed aggregates of ca. 100- to 200-μm diameter (Fig. 2a) in which *Methanosarcina* and *Geobacter* were intertwined (Fig. 2b).

Electrical connections via outer surface proteins and granular activated carbon. Previous studies have demonstrated that deleting *pilA*, which encodes the structural protein of the conductive pili of *Geobacter* species, eliminates the ability of *G. metallireducens* to participate in DIET (15, 21). Cocultures initiated with a *pilA*-deficient strain (33) of *G. metallireducens* failed to metabolize ethanol or produce methane (Fig. 3a). Furthermore, cocultures initiated with a strain of *G. metallireducens* in which a gene (Gmet 1868 gene) for an outer surface cytochrome required for Fe(III)

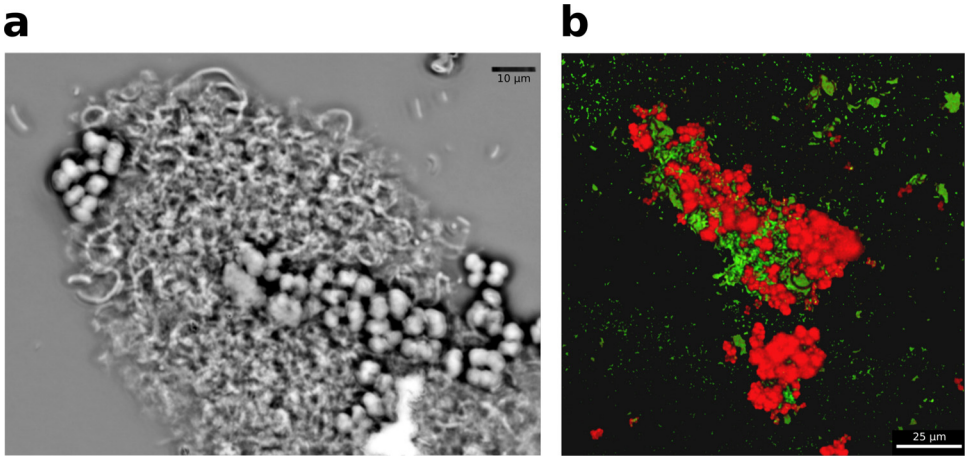

FIG 2 Coculture composition. (a) Phase-contrast micrograph of an aggregate of *M. barkeri* (cocci) and *G. metallireducens* (rods). (b) Scanning laser confocal microscope image of coculture aggregate after *in situ* hybridization, which targeted *Methanosarcina* cells with a red probe (Cy3) and *G. metallireducens* cells with a green probe (Cy5). Images are representative of triplicate samples taken during the mid-exponential growth phase of the cocultures.

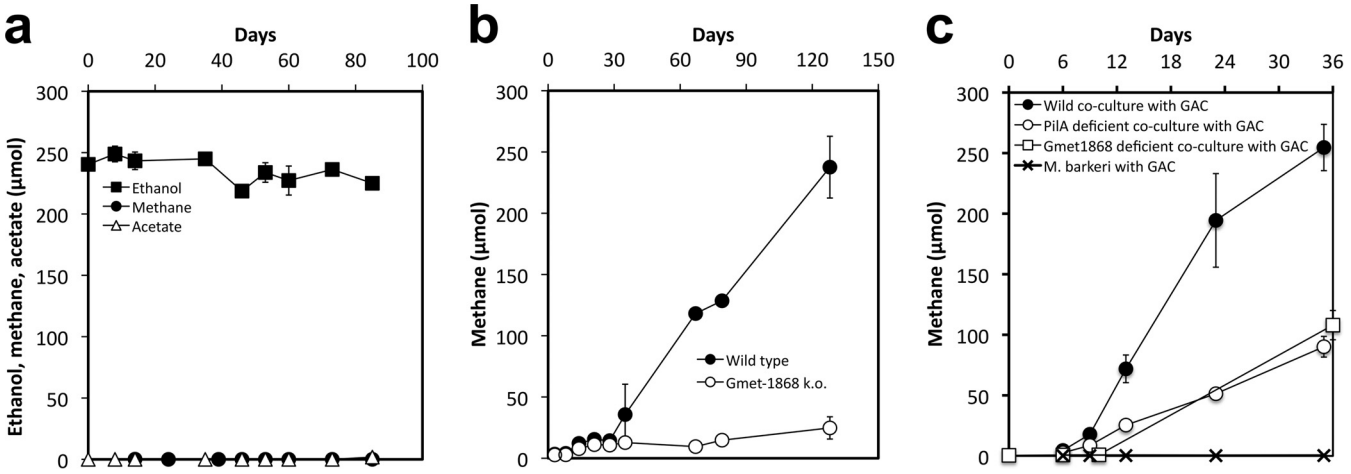

FIG 3 Mutant studies. (a) Lack of ethanol metabolism and methane production in a coculture initiated with a *pilA*-deficient *G. metallireducens* strain. (b) Methane production in cocultures initiated with wild-type *G. metallireducens* or a strain deficient in the Gmet 1868 gene, which encodes a predicted extracellular cytochrome. (c) GAC stimulation of methane production in cocultures initiated with wild-type *G. metallireducens* and enabling methane production in cocultures initiated with either the PilA- or cytochrome (Gmet 1868)-deficient strains of *G. metallireducens*. All incubations were set up with a 5% (vol/vol) inoculum of each coculture partner.

oxide reduction was deleted (34) also failed to metabolize ethanol to methane (Fig. 3b).

However, cocultures initiated with either the *pilA*-deficient or Gmet 1868-deficient strains of *G. metallireducens* did effectively produce methane in the presence of granular activated carbon (GAC) (Fig. 3c). This is consistent with previous studies that demonstrated that GAC, which is electrically conductive, served as an electrical conduit for DIET between *G. metallireducens* and *G. sulfurreducens*, permitting the two species to share electrons even when the coculture was initiated with strains of *G. sulfurreducens* that could not produce pili or the pilin-associated *c*-type cytochrome OmcS (32). As previously noted (32), GAC amendments also stimulated the conversion of ethanol to methane in cocultures initiated with wild-type *G. metallireducens* and *M. barkeri* (Fig. 3c), substantially reducing the 39-day lag phase observed in the absence of GAC (Fig. 1a). Methane production of the cocultures initiated with the *pilA*-deficient or Gmet 1868-deficient strains of *G. metallireducens* was slower than that of the wild-type cocultures amended with GAC, but even in the absence of pili or the outer surface *c*-type cytochrome, methane production rates in the presence of GAC required a <6-day lag period to initiate ethanol metabolism (Fig. 3b). Previous studies demonstrated that in cocultures of *G. metallireducens* with either *M. barkeri* or *G. sulfurreducens*, both syntrophic partners attached to the GAC surface, but the individual cells were too far separated for biological electrical connections between the species (32). These results suggest that GAC can substitute for pili and/or outer surface cytochromes as the electrical conduit between the electron-donating *G. metallireducens* and the electron-accepting *M. barkeri*.

Growth of *M. barkeri* via HIT with *P. carbinolicus*. Like *G. metallireducens*, *P. carbinolicus* can grow on ethanol in the presence of a syntrophic partner, but it relies on HIT rather than DIET (20, 43). *P. carbinolicus* and *M. barkeri* grew in coculture in medium with ethanol as the sole electron donor, but unlike *G. metallireducens-M. barkeri* cocultures, there was a steady accumulation of acetate (Fig. 4 left panel). This was probably due to the fact that acetate metabolism in *M. barkeri* is repressed when H_2 is available (44–46).

P. carbinolicus metabolizes ethanol to acetate and H_2 according to the following equation (20): $2\ C_2H_6O + 2\ H_2O \rightarrow 2\ C_2H_4O_2 + 4\ H_2$. The metabolism of ethanol to acetate and H_2 is exergonic only if *M. barkeri* can oxidize H_2 with the reduction of carbon dioxide as follows: $4\ H_2 + CO_2 \rightarrow CH_4 + H_2O$.

The stoichiometry of ethanol consumed (mean ± standard deviation, 0.91 ± 0.07 mmol; $n = 4$), methane produced (0.41 ± 0.07 mmol), and acetate accumulated (0.66 ± 0.07 mmol) was consistent with methane being produced almost exclusively from H_2 oxidation with the reduction of carbon dioxide, assuming that some of the acetate produced was diverted to biomass production. A similar partial oxidation of ethanol with the accumulation of acetate was observed in the ethanol-metabolizing cocultures of *P. carbinolicus* and *G. sulfurreducens*, which were dependent on HIT (20), whereas acetate did not accumulate in *G. metallireducens-G. sulfurreducens* cocultures that relied on DIET (20).

In contrast to the *G. metallireducens-M. barkeri* cocultures (Fig. 2a and b), the *P. carbinolicus-M. barkeri* cocultures did not form multispecies aggregates (Fig. 4b and c). In a similar manner, *P. carbinolicus-G. sulfurreducens* cocultures did not aggregate (20), whereas *G. metallireducens-G. sulfurreducens* cocultures did (19). These results are consistent with the need for direct cell-to-cell contact for DIET, but not for HIT.

Implications. The results demonstrate that *M. barkeri* is able to participate in DIET. It is only the second methanogen found to have this capability. *Methanosarcina* species are important contributors to methane production in some anaerobic digesters (29) as well as soils and sediments (7–10). Thus, the potential for DIET should be considered when attempting to promote the growth of *Methanosarcina* in digesters or when modeling their activity in soils and sediments.

In previous pure-culture studies, *Methanosaeta harundinacea*, which is incapable of utilizing H_2 as an electron donor, was found to be capable of DIET, but the two known H_2-utilizing methanogens tested, *Methanospirillum hungatei* and *Methanobacterium formicicum*, were not (15). Thus, *M. barkeri* is more versatile than other methanogens that have been evaluated for DIET, with the capacity to either accept electrons in the form of H_2 from syn-

FIG 4 Syntrophic growth in cocultures of *P. carbinolicus* and *M. barkeri*. (Left) Syntrophic growth in the third transfer of the coculture. (Center) Phase-contrast image illustrating the lack of aggregation of *P. carbinolicus* (rods) and *M. barkeri* (cocci). (Right) Scanning laser confocal microscope image of coculture after *in situ* hybridization, which targeted *Methanosarcina* cells with a red probe (Cy3) and *P. carbinolicus* cells with a green probe (Cy5). Images are representative of triplicate samples taken during the mid-exponential growth phase of the cocultures.

tropic partners like *Desulfovibrio* (46) and *Pelobacter* (this study) or to participate in DIET.

In the studies on DIET with *M. harundinacea*, acetate was available as an additional substrate for methane production (15), as it was in the *M. barkeri* cocultures reported here. However, studies with cocultures of *G. metallireducens* and a strain of *G. sulfurreducens* unable to oxidize acetate demonstrated that electrons derived from DIET could support fumarate reduction and growth of *G. sulfurreducens* in the absence of acetate as an additional electron donor (22). Similar studies should be feasible with an *M. barkeri* mutant unable to metabolize acetate (47) to determine whether carbon dioxide reduction to methane with electrons derived from DIET provides sufficient energy to support cell maintenance and growth. Other than the obvious importance of DIET in some anaerobic digesters treating brewery wastes (15, 18), the prevalence of DIET in other methanogenic communities is unknown. The fact that at least two major genera of methanogens have evolved the capacity for DIET suggests that there are conditions in some soils and sediments in which DIET confers a selective advantage.

The ability of GAC to promote methanogenic DIET has important implications for the design of anaerobic digesters. Previous studies that have reported stimulation in methane production when carbon cloth was introduced into digesters have attributed this response to the cloth retaining methanogens (48–50). However, the possibility that these conductive materials might promote DIET should also be considered.

Although the importance of electrically conductive pili and outer surface cytochromes in extracellular electron exchange, including DIET, is becoming increasingly apparent for *Geobacter* species (51, 52), the potential extracellular electron contacts that might permit methanogens to accept electrons via DIET are less clear. *Methanosaeta* and *Methanosarcina* are the only genera of methanogens with membrane-bound cytochromes (53, 54) that could potentially play a role in extracellular electron exchange. The availability of tools for genetic manipulation of *M. barkeri* (55) suggests that it may be the ideal candidate for functional analysis of DIET mechanisms in methanogens.

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